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Antiparasitic activities of novel pyrimidine *N*-acylhydrazone hybrids

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Abstract

In this article, a series of 29 new pyrimidine *N*-acylhydrazone hybrids were synthesized and evaluated in vitro against *Leishmania amazonensis* and *Trypanosoma cruzi* protozoa that cause the neglected diseases cutaneous leishmaniasis and Chagas disease, respectively. Eight of the target compounds showed significant antiprotozoal activities with IC_{50} values in 4.3–33.6 μM range. The more active compound **4f** exhibited selectivity index greater than 15 and drug-like properties based on Lipinski's rule.

KEYWORDS

Chagas disease, *Leishmania amazonensis*, leishmaniasis, *N*-acylhydrazone, pyrimidine, *Trypanosoma cruzi*

1 | INTRODUCTION

Leishmaniasis is an endemic disease and considered neglected tropical disease (NTD), that is present in 98 countries on five continents, with a marked occurrence in underdeveloped and developing countries with a subtropical climate. This disease affects more than 12 million people, with approximately 1 million new cases per year. It is a chronic disease caused by flagellated protozoa of the genus *Leishmania*, transmitted to mammals by the bite of infected sand flies. The clinical manifestation depends mainly on the *Leishmania* species involved. Cutaneous leishmaniasis is the most common, while visceral leishmaniasis is the most serious form. The drugs used nowadays in the treatment of leishmaniasis are expensive and have shown highly toxic effects and parasite resistance (Sangshetti, Khan, Kulkarni, Arote, & Patil, 2015; WHO, 2010).

Another neglected disease that causes more than 10,000 deaths per year is Chagas disease (CD). This disease is endemic in Latin America but now expanding over the globe. It is estimated that 8 million people are infected with the protozoan parasite *Trypanosoma cruzi*, transmitted by "kissing bugs," and more than 25 million people risk acquiring the disease. Furthermore, CD can be transmitted orally by contaminated food/beverage consumption (Coura, 2015). Currently, there are only two drugs used for treatment and their effectiveness depends on the stage of the disease. In cases of more advanced infection, the effectiveness of medications may be reduced. In addition, there are reports of side effects that lead to treatment abandonment (WHO, 2010).

Thus, considering the ineffective treatments available and the lack of interest by pharmaceutical companies for theses NTD, there is a clear need for research and development of new drugs capable of providing adequate treatments for these diseases.

Aza-heterocycle compounds are known for the potent pharmacology properties and their wide application in medicinal chemistry. In particular, heterocycle-*N*-acylhydrazone hybrids have exhibited important pharmacological activity, such as antileishmanial effects (Bernardino et al., 2006). Thus, our research group has focused on the development of methodologies for the synthesis of these hybrids and evaluated their antileishmanial effects. We reported the synthesis and in vitro antileishmanial activity of a series of 1,4,6-trisubstituted pyrazolo[3,4-*d*]pyridazin-7-one-*N*-acylhydrazone hybrids forward promastigote and amastigote forms of *Leishmania amazonensis* (Jacomini et al., 2016; Jacomini et al., 2018). Hybrids **I**, **II**, and **III** exhibited good activity values against both forms of *L. amazonensis*, with excellent values for compound **II** ($IC_{50(pro)}$ = 3.63 μM, IC_{50}

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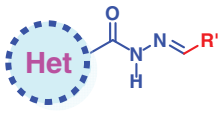
$_{(ama)}$ = 2.32 μM) and remarkable selectivity index (SI > 52.71 and 82.48) (Figure 1a). Recently, Coimbra et al. reported the antileishmanial activity of pyrimidine *N*-acylhydrazone hybrids. Five compounds were active against *L. amazonensis* on promastigote and amastigote forms, with the best results for compounds **IV** ($IC_{50(pro)}$ = 27.42 μM, $IC_{50(ama)}$ = 33.91 μM) and **V** ($IC_{50(pro)}$ = 24.28 μM, $IC_{50(ama)}$ = 10.8 μM) (Figure 1a). Both compounds caused mitochondrial depolarization and increased of intracellular reactive oxygen species levels (Coimbra, de Souza, Terror, Pinheiro, & Granato, 2019).

Regarding the search for actives molecules against Chagas disease, heterocycle-*N*-acylhydrazone hybrids also have shown promising results. Vera-DiVaio et al. published the synthesis and in vitro trypanocidal evaluation studies of a new series of *N*-phenylpyrazole benzylidene-*N*-acylhydrazone (**VI** and **VII**, Figure 1). Twenty-six compounds showed good trypanocidal activity, with the best results for compounds **VI** (IC_{50} = 30 μM) and **VII** (IC_{50} = 50 μM) (Figure 1b) (Vera-DiVaio et al., 2009).

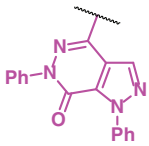
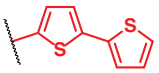
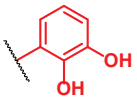
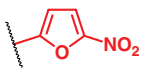
Encouraged by these observations and in continuation of our efforts directed toward the synthesis of new aza-heterocycle derivatives containing an *N*-acylhydrazone subunit, we report herein the synthesis of a new series of 2-substituted-pyrimidine-*N*-acylhydrazone hybrids (Scheme 1) and the evaluation of their antileishmanial and anti-*Trypanosoma cruzi* profile.



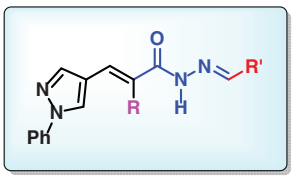
(Heterocyle *N*-acylhydrazone hybrids)

(a) *Jacomini et al.*

(b) *Coimbra et al.*

compd	Het	R'	IC_{50}(μM) promastigote	IC_{50}(μM) amastigote
I			84.96±4.9 (SI 1.25)	32.71±7.4 (SI 3.25)
II			3.63±1.24 (SI >52.71)	2.32±0.45 (SI >82.48)
III			16.2±2.6 (SI 14.2)	3.84±2.1 (SI 59.9)
IV			27.42±0.95 (SI >5.47)	33.91±0.81 (SI >4.42)
V			24.28±0.9 (SI 1.19)	10.8±0.63 (SI 2.68)

(c) *VeraDiVaio et al.*



(*N*-acylhydrazone derivatives)

compd	R	R'	IC_{50}(μM)	T_{ox}(%)
VI	Ph	4-$NO_2C_6H_4$	30	19.4
VII	4-FC_6H_4	4-CNC_6H_4	50	19.4

(d) *This work*

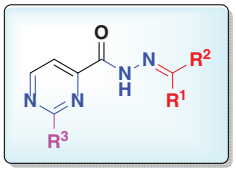
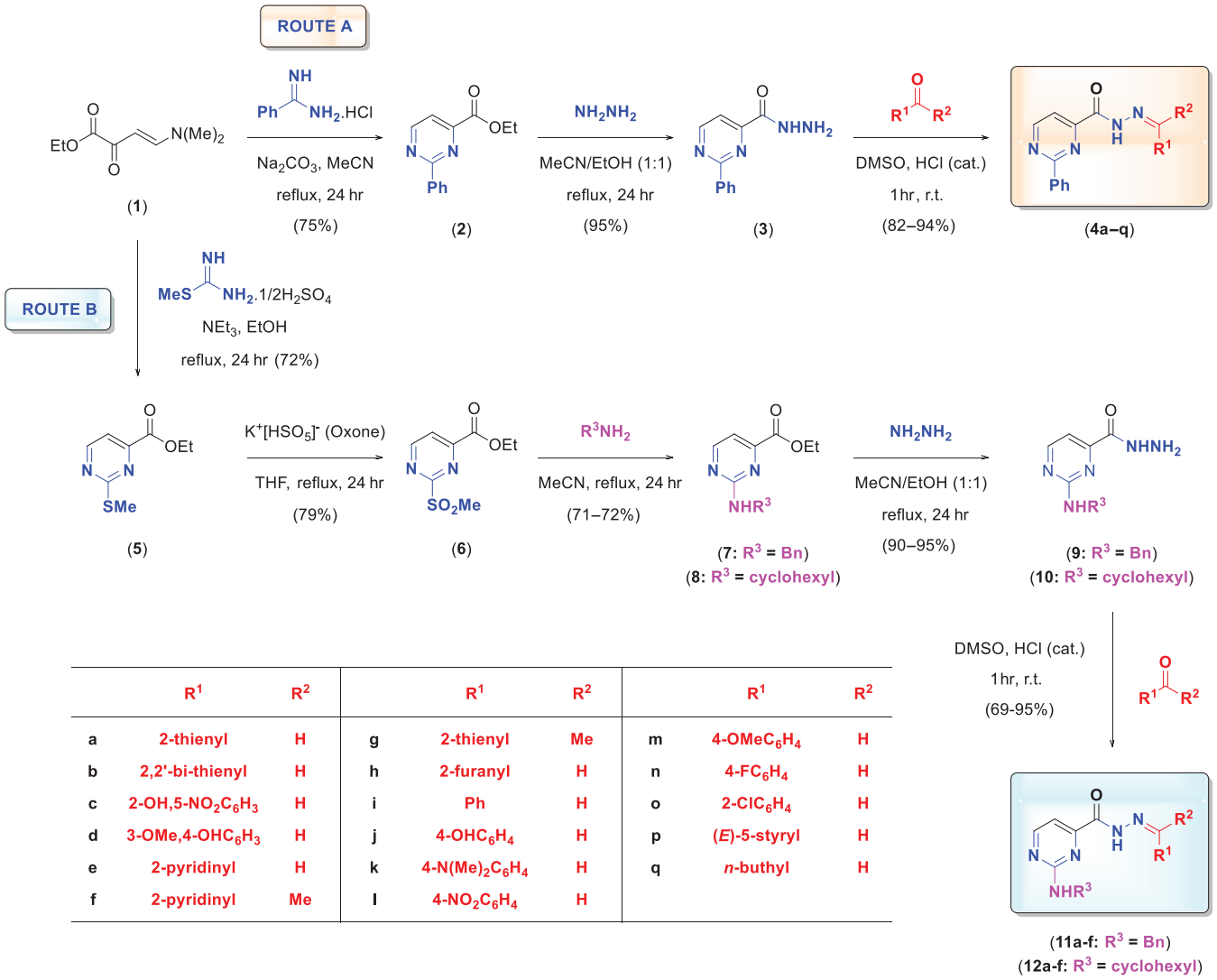


FIGURE 1 Heterocycle-*N*-acylhydrazone hybrids and their antiprotozoal activities (a–c); molecule designed for this work (d)



	R^1	R^2		R^1	R^2		R^1	R^2
a	2-thienyl	H	g	2-thienyl	Me	m	4-OMeC_6H_4	H
b	2,2'-bi-thienyl	H	h	2-furanyl	H	n	4-FC_6H_4	H
c	2-OH,5-$NO_2C_6H_3$	H	i	Ph	H	o	2-ClC_6H_4	H
d	3-OMe,4-OHC_6H_3	H	j	4-OHC_6H_4	H	p	(*E*)-5-styryl	H
e	2-pyridinyl	H	k	4-N(Me)$_2C_6H_4$	H	q	*n*-buthyl	H
f	2-pyridinyl	Me	l	4-$NO_2C_6H_4$	H			

SCHEME 1 Synthetic routes (a and b) for target 2-substituted-pyrimidine-*N*-acylhydrazone hybrid compounds (**4a–q**, **11a–f**, and **12a–f**)

2 | MATERIALS AND METHODS

2.1 | Chemicals and instruments

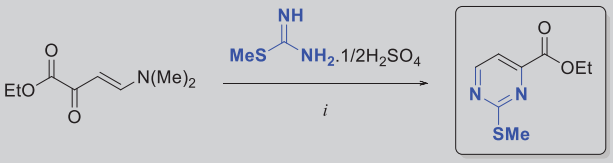
Reagents were used as obtained from commercial suppliers without further purification. The reactions were monitored by thin-layer chromatography using Merck TLC silica gel plates and visualized with ultraviolet (UV) light. The column chromatography used was silica gel 60, with 230–400 mesh (Merck). All melting points were measured with MQAPF-307 Microquímica apparatus using benzoic acid as internal standard. ^{1}H and ^{13}C NMR, HSQC, and HMBC experiments were run on Bruker Avance III HD apparatus operating at ^{1}H 300.06 MHz and ^{13}C 75.46 MHz, and Bruker Avance III HD apparatus operating at ^{1}H 500.13 MHz and ^{13}C 125.77 MHz. Chemical shifts are reported in ppm using TMS as the internal standard for $CDCl_3$ and DMSO-d_6. Electrospray ionization ESI(+)-MS and tandem ESI(+)-MS/MS were acquired using a hybrid high-resolution and high accuracy microTof (Q-TOF) mass spectrometer (Bruker). For ESI(+)-MS, the energy for the collision induced dissociations (CDI) was optimized for each component. For data acquisition and processing, the Q-TOF-control data analysis software (Bruker Scientific) was used.

2.2 | Procedure for the synthesis of pyrimidine 2

A mixture of the β-enamino ketone **1** (1 mmol, 0.171 g), benzamidine hydrochloride (1.2 mmol, 0.188 g), and sodium carbonate (1.2 mmol, 1.2 equiv, 0.127 g) in acetonitrile (15 ml) was stirred under reflux for 24 hr. Next, water (25 ml) was added and the mixture was extracted with dichloromethane (3 × 20 ml). The organic phase was dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure and the obtained residue was purified on a silica gel chromatography column, utilizing an 80:20 mixture of hexane:ethyl acetate as the eluent.

4-(Ethoxycarbonyl)-2-phenylpyrimidine (2) is a crystalline light yellow solid; 75% yield (0.171 g); mp 58.4–60.9; ^{1}H NMR (500.13 MHz,

TABLE 1 Reaction optimization for the construction of pyrimidine core **5**



(**1**) (**5**)

	i				
Entry	**Solvent**	**Base**	**Temp**	**Time (hr)**	**Conversion (%)[a]**
1	H_2O	Na_2CO_3 (2 equiv)	r.t.	3	0
2	MeCN	Na_2CO_3 (1.2 equiv)	Reflux	8	43
3	MeCN	Na_2CO_3 (2 equiv)	Reflux	24	52
4	MeCN	Na_2CO_3 (4 equiv)	Reflux	24	63
5	MeCN	DBU (2 equiv)	Reflux	24	30
6	EtOH	Na_2CO_3 (4 equiv)	Reflux	24	70
7	EtOH	NEt_3 (5 equiv)	Reflux	24	90 (72)[b]

[a]Conversion calculated from the ^{1}H NMR spectrum of the crude product.
[b]Yield after purification by column chromatography.

$CDCl_3$) δ (ppm) 1.48 (t, 3H, *J* = 7.1 Hz, O–CH_2–CH_3), 4.52 (q, 2H, *J* = 7.1 Hz, O–CH_2–CH_3), 7.50–7.53 (m, 3H, Ph), 7.84 (d, 1H, *J* = 4.9 Hz, H6), 8.52–8.54 (m, 2H, Ph), 9.02 (d, 1H, *J* = 4.9 Hz, H5); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ (ppm) 14.3 (O–CH_2–CH_3), 62.5 (O–CH_2–CH_3), 118.4 (C6), 128.6, 128.7, 131.3, 136.9 (Ph), 155.6 (C4), 159.3 (C5), 164.5 (C2), 165.3 (C5'); HRMS (ESI+): calcd for $C_{13}H_{13}N_2O_2^+$, $[M + H]^+$: 229.0972, found 229.0980.

2.3 | Procedure for the synthesis of pyrimidine 5

To a solution of *β*-enamino ketone **1** (1 mmol, 0.171 g), in ethanol (15 ml), was added *S*-methylisothiourea hemisulfate salt (2 mmol, 0.278 g) and triethylamine (5 mmol, 0.506 g). The reaction mixture was stirred under reflux for 24 hr. Then, the solvent was evaporated under reduced pressure and the reaction mixture was washed with water (25 ml), extracted with dichloromethane (3 × 20 ml) and the organic phase was dried over anhydrous sodium sulfate. The solvent was again evaporated under reduced pressure and the obtained residue was purified on a silica gel chromatography column, utilizing a 75:15 mixture of hexane:ethyl acetate as the eluent.

4-(Ethoxycarbonyl)-2-(methylthio)pyrimidine (5) is an yellow liquid, 72% yield (0.143 g); ^{1}H NMR (300.06 MHz, $CDCl_3$) δ (ppm) 1.43 (t, 3H, *J* = 7.1 Hz, O–CH_2–CH_3), 2.62 (s, 3H, H4'), 4.46 (q, 2H, *J* = 7.1 Hz, O–CH_2–CH_3), 7.59 (d, 1H, *J* = 4.9 Hz, H6), 8.72 (d, 1H, *J* = 4.9 Hz, H5); ^{13}C NMR (75.46 MHz, $CDCl_3$) δ (ppm) 14.3 (O–CH_2–CH_3, C4'), 62.6 (O–CH_2–CH_3), 115.6 (C6), 155.5 (C4), 159.1 (C5), 164.1 (C5'), 174.1 (C2); HRMS (ESI+): calcd for $C_8H_{11}N_2O_2S^+$, $[M + H]^+$: 199.0536, found 199.0547.

2.4 | Procedure for the synthesis of pyrimidine 6

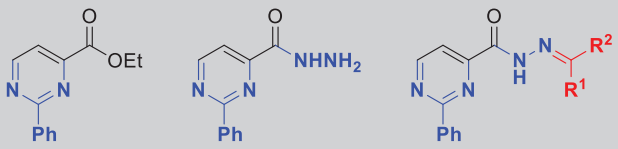
A mixture of the intermediate **5** (1 mmol, 0.198 g) and potassium peroxymonosulfate (Oxone) (3 mmol, 0.922 g) in THF (20 ml), was stirred under reflux for 24 hr. Then, the solvent was evaporated under reduced pressure and the reaction mixture was washed with distilled water (25 ml) and was extracted with dichloromethane (3 × 20 ml). The organic phase was dried over anhydrous sodium sulfate and the solvent was again evaporated under reduced pressure. The compound **6** was obtained pure without further purification. Yellow liquid, 79% yield (0.182 g).

4-(Ethoxycarbonyl)-2-(methylsulfonyl)pyrimidine (6) is an yellow liquid, 79% yield (0.182 g); ^{1}H NMR (500.13 MHz, DMSO-d_6) δ (ppm) 1.46 (t, 3H, *J* = 7.1 Hz, O–CH_2–CH_3), 3.44 (s, 3H, H4'), 4.52 (q, 2H, *J* = 7.1 Hz, O–CH_2–CH_3), 8.21 (d, 1H, *J* = 4.9 Hz, H6), 9.19 (d, 1H, *J* = 5.0 Hz, H5); ^{13}C NMR (125.77 MHz, DMSO-d_6) δ (ppm); HRMS (ESI+): calcd for $C_8H_{11}N_2O_4S^+$, $[M + H]^+$: 231.0434, found 231.0441.

2.5 | General procedure for the synthesis pyrimidine derivatives 7 and 8

To a solution of compound **6** (1 mmol, 0.230 g), in acetonitrile (15 ml), was added benzylamine (3 mmol, 0.321 g) or cyclohexylamine (3 mmol, 0.298 g) and the mixture was stirred under reflux for 2 hr (for benzylamine) or 10 hr (for cyclohexylamine). Then, the reaction solvent was evaporated under reduced pressure and the crude compound was solubilized in chloroform. Next, it was washed with an acid solution (HCl, 1 M aq.; 20 ml), extracted with chloroform (3 × 20 ml)

TABLE 2 IC_{50} and CC_{50} values for pyrimidines **2**, **3**, and **4a**–**q** against *L. amazonensis* and *T. cruzi*



(2) (3) (4a-q)

Comp.	R^1	R^2	IC_{50} (μM)		CC_{50} (μM)		
			L. amazonensis (pro.)	*T. cruzi* (epimas.)	$LLCMK_2$[a]	L929[b]	J774A1[c]
2	–	–	>100	>100	N/A	N/A	>100
3	–	–	>100	>100	N/A	N/A	>100
4a	2-Thienyl	H	>100	>100	N/A	N/A	>100
4b	2,2′-Bi-thienyl	H	12.0 ± 1.4	N/A	N/A	N/A	>100
4c	2-OH,5-$NO_2C_6H_3$	H	32.9 ± 5.0	17.2 ± 1.8	87.2 ± 1.1	323.5 ± 16.5	76.0 ± 3,0
4d	3-OMe,4-OHC_6H_3	H	153.0 ± 19.0	67.9 ± 2.9	96.0 ± 1.6	452.5 ± 2.5	65.3 ± 5.8
4e	2-Pyridinyl	H	177.6 ± 8.6	>200	>1,000	802.5 ± 12.5	409.5 ± 3.1
4f	2-Pyridinyl	Me	4.3 ± 0.3	>200	>1,000	65.0 ± 2.1	>1,000
4g	2-Thienyl	Me	>200	>200	>1,000	>1,000	876.5 ± 5.9
4h	2-Furanyl	H	>200	>200	>1,000	>1,000	>1,000
4i	Ph	H	>200	>200	>1,000	>1,000	>1,000
4j	4-OHC_6H_4	H	>200	>200	>1,000	>1,000	>1,000
4k	4-$N(Me)_2C_6H_4$	H	>200	176.5 ± 5.9	308.6 ± 5.6	>1,000	128.5 ± 1.8
4l	4-$NO_2C_6H_4$	H	>200	33.6 ± 1.3	287.5 ± 1.9	>1,000	198.4 ± 3.2
4m	4-$OMeC_6H_4$	H	>200	65.9 ± 1.4	98.5 ± 2.3	>1,000	68.0 ± 1.9
4n	4-FC_6H_4	H	>200	108.5 ± 2.8	297.0 ± 5.4	>1,000	115.9 ± 2.8
4o	2-ClC_6H_4	H	>200	95.3 ± 3.6	176.0 ± 4.9	>1,000	194.3 ± 2.0
4p	(*E*)-5-Styryl	H	>200	51.4 ± 5.3	165.9 ± 5.4	>1,000	60.4 ± 3.2
4q	*n*-Butyl	H	>200	> 200	>1,000	>1,000	>1,000

[a]Cytotoxic concentration corresponding to 50% inhibition of epithelial cells.
[b]Cytotoxic concentration corresponding to 50% inhibition of fibroblast.
[c]Cytotoxic concentration corresponding to 50% inhibition of macrophage growth.

and the organic phase was dried over anhydrous sodium sulfate. Subsequently, the solvent was again evaporated under reduced pressure and the compound was purified on a silica gel chromatographic column, using a 75:15 mixture of hexane:ethyl acetate as eluent.

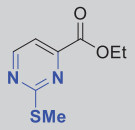
4-(Ethoxycarbonyl)-2-(benzylamino)pyrimidine (7) is a white solid; 71% yield (0.185 g); mp 76.0–77.2; ^{1}H NMR (500.13 MHz, $CDCl_3$) δ (ppm) 1.40 (t, 3H, *J* = 7.1 Hz, O–CH_2–CH_3), 4.42 (q, 2H, *J* = 7.2 Hz, O–CH_2–CH_3), 4.68 (d, 2H, *J* = 5.9 Hz, H4′), 5.98 (s, 1H, H3′), 7.18 (d, 1H, *J* = 4.9 Hz, H6), 7.24–7.27 (m, 1H, Ph), 7.30–7.36 (m, 4H, Ph), 8.45 (s, 1H, H5); ^{13}C NMR (125.77 MHz, $CDCl_3$) δ (ppm) 14.3 (O–CH_2–CH_3), 45.7 (C4′), 62.3 (O–CH_2–CH_3), 110.2 (C6), 127.4, 127.7, 128.7, 138.8 (Ph), 156.2 (C4), 160.3 (C5), 162.7 (C5′), 164.7 (C2); HRMS (ESI+): calcd for $C_{14}H_{16}N_3O_2^+$, $[M + H]^+$: 258.1237, found 258.1246.

4-(Ethoxycarbonyl)-2-(cyclohexylamino)pyrimidine (8) is a light yellow solid, 72% yield (0.182 g); mp 53.7–54.8; ^{1}H NMR (300.06 MHz, $CDCl_3$) δ (ppm) 1.12–1.24 (m, 3H, cyclohexyl), 1.31–1.43 (m, 2H, cyclohexyl), 1.36 (t, 3H, *J* = 7.1 Hz, O–CH_2–CH_3), 1.55–1.62 (m, 1H, cyclohexyl), 1.70 (dt, 2H, *J* = 12.9, 4.0 Hz, cyclohexyl), 1.98 (dq, 2H, *J* = 12.4, 4.0 Hz, cyclohexyl), 3.82 (qt, 1H, *J* = 10.0, 7.8, 3.7 Hz, H4′), 4.39 (q, 2H, *J* = 7.1 Hz, O–CH_2–CH_3), 5.34 (d, 1H, *J* = 8.2 Hz, H3′), 7.08 (d, 1H, *J* = 4.9 Hz, H6), 8.43 (d, 1H, *J* = 4.9 Hz, H5); ^{13}C NMR (75.46 MHz, $CDCl_3$) δ (ppm) 14.2 (O–CH_2–CH_3), 24.8, 25.7, 33.0, 49.8 (cyclohexyl), 62.2 (O–CH_2–CH_3), 109.4 (C6), 156.1 (C4), 160.2 (C5), 162.1 (C5′), 164.7 (C2); HRMS (ESI+): calcd for $C_{13}H_{20}N_3O_2^+$, $[M + H]^+$: 250.1550, found 250.1562.

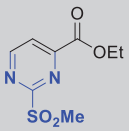
2.6 | General procedure for the synthesis of pyrimidine derivatives 3, 9, and 10

A mixture of the ester derivative (1 mmol, **2**: 0.228 g; **7**: 0.257 g; **8**: 0.249 g) with hydrazine monohydrate 80% (20 mmol, 1 g), in EtOH/MeCN (1:1) (25 ml), was stirred under reflux for 24 hr. Then, it was

TABLE 3 IC_{50} and CC_{50} values for pyrimidines **5**–**10**, **11a**–**f**, and **12a**–**f** against *L. amazonensis* and *T. cruzi*



(5)



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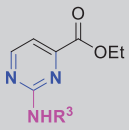
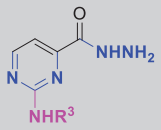
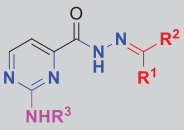


(7: R^3 = Bn)
(8: R^3 = cyclohexyl)



(9: R^3 = Bn)
(10: R^3 = cyclohexyl)



(11a-f: R^3 = Bn)
(12a-f: R^3 = cyclohexyl)

			IC_{50} (μM)		CC_{50} (μM)		
Comp.	R^1	R^2	*L. amazonensis* (pro.)	*T. cruzi* (epimas.)	$LLCMK_2$[a]	L929[b]	J774A1[c]
5	–	–	22.1 ± 1.5	28.7 ± 2.8	90.5 ± 1.8	67.8 ± 1.0	75.1 ± 2.9
6	–	–	15.9 ± 2.0	16.9 ± 0.8	87.0 ± 3.7	70.3 ± 2.9	69.1 ± 3.2
7	–	–	14.3 ± 1.7	19.5 ± 2.6	91.4 ± 1.9	68.5 ± 5.1	77.3 ± 2.0
8	–	–	19.6 ± 2.3	29.0 ± 3.1	98.5 ± 2.8	76.4 ± 1.8	62.6 ± 1.8
9	–	–	133.6 ± 12.1	167.9 ± 9.0	>1,000	>1,000	>1,000
10	–	–	112.0 ± 8.4	148.2 ± 12.1	891.6 ± 12.9	733.1 ± 2.0	702.6 ± 3.9
11a	2-Thienyl	H	>200	>200	>1,000	>1,000	>1,000
11b	2,2′-Bi-thienyl	H	68.4 ± 7.8	97.3 ± 2.0	475.8 ± 3.7	387.5 ± 7.9	372.1 ± 3.3
11c	2-OH,5-$NO_2C_6H_3$	H	18.9 ± 3.0	22.3 ± 0.9	431.2 ± 1.9	391.0 ± 0.8	333.8 ± 9.7
11d	3-OMe,4-OHC_6H_3	H	77.1 ± 10.4	79.8 ± 9.5	654.6 ± 2.8	601.7 ± 8.0	532.9 ± 8.1
11e	2-Pyridinyl	H	53.9 ± 2.1	61.3 ± 1.9	519.3 ± 6.2	502.6 ± 2.1	411.6 ± 5.0
11f	2-Pyridinyl	Me	25.2 ± 2.3	29.7 ± 0.6	572.4 ± 7.6	569.9 ± 11.5	498.3 ± 1.7
12a	2-Thienyl	H	>200	>200	>1,000	>1,000	>1,000
12b	2,2′-Bi-thienyl	H	25.0 ± 3.9	47.1 ± 2.0	128.9 ± 5.4	115.0 ± 2.5	98.1 ± 3.6
12c	2-OH,5-$NO_2C_6H_3$	H	56.7 ± 3.9	74.0 ± 7.9	878.5 ± 12.7	702.6 ± 2.4	619.5 ± 2.4
12d	3-OMe,4-OHC_6H_3	H	44.9 ± 5.0	61.2 ± 2.8	692.3 ± 11.5	605.0 ± 9.4	587.1 ± 1.6
12e	2-Pyridinyl	H	184.6 ± 5.4	>200	>1,000	>1,000	914.8 ± 11.9
12f	2-Pyridinyl	Me	23.7 ± 0.6	63.7 ± 10.6	409.1 ± 4.4	386.8 ± 3.4	311.4 ± 7.9

[a]Cytotoxic concentration corresponding to 50% inhibition of epithelial cells.
[b]Cytotoxic concentration corresponding to 50% inhibition of fibroblast.
[c]Cytotoxic concentration corresponding to 50% inhibition of macrophage growth.

precipitated with the addition of cold EtOH, filtered and dried under vacuum.

4-(Carbohydrazide)-2-phenylpyrimidine (3) is a white solid; 95% yield (0.203 g); mp 118.4–119.7; ^{1}H NMR (500.13 MHz, DMSO-d_6) δ (ppm) 4.76 (bs, 2H, NH_2), 7.52–7.58 (m, 3H, Ph), 7.87 (d, *J* = 4.9 Hz, H6), 8.65–8.67 (m, 2H, Ph), 9.10 (d, 1H, *J* = 4.9 Hz, H5), 10.52 (bs, 1H, NH); ^{13}C NMR (125.77 MHz, DMSO-d_6) δ (ppm) 116.2 (C6), 128.4, 128.6, 131.2. 136.4 (Ph), 156.9 (C4), 159.9 (C5), 161.0 (C5'), 162.9 (C2); HRMS (ESI+): calcd for $C_{11}H_{11}N_4O^+$, $[M + H]^+$: 215.0927, found 215.0941.

4-(Carbohydrazide)-2-(benzylamino)pyrimidine (9) is a white solid; 95% yield (0.231 g); mp 171.7–172.5; ^{1}H NMR (300.06 MHz, DMSO-d_6) δ (ppm) 4.62 (d, 2H, *J* = 13.8 Hz, H4'), 4.64 (bs, 2H, H7'), 7.02 (d, 1H, *J* = 4.8 Hz, H6), 7.18–7.23 (m, 1H, Ph), 7.27–7.36 (m, 4H, Ph), 7.89 (bs, 1H, H3'), 8.46 (d, 1H, *J* = 4.8 Hz, H5), 9.83 (bs, 1H, H6'); ^{13}C NMR (75.46 MHz, DMSO-d_6) δ (ppm) 43.8 (C4'), 106.6 (C6), 126.6, 127.3, 128.2, 140.3 (Ph), 157.0 (C4), 160.4 (C5), 161.8 (C5'), 161.9 (C2); HRMS (ESI+): calcd for $C_{12}H_{14}N_5O^+$, $[M + H]^+$: 244.1193, found 244.1202.

4-(Carbohydrazide)-2-(cyclohexylamino)pyrimidine (10) is a white solid; 90% yield (0.211 g); mp 157.8–158.9; ^{1}H NMR (500.13 MHz, DMSO-d_6) δ (ppm) 1.11–1.15 (m, 1H, cyclohexyl), 1.19–1.25 (m, 2H, cyclohexyl), 1.33–1.34 (m, 2H, cyclohexyl), 1.58 (dt, 1H, *J* = 13.0, 3.5 Hz, cyclohexyl), 1.68 (dt, 2H, *J* = 13.1, 3.7 Hz, cyclohexyl), 1.84 (dd, 2H, *J* = 12.7, 4.6 Hz, cyclohexyl), 3.38 (bs, 2H, H7'), 3.86 (d, 1H, *J* = 119.7 Hz, H4'), 4.61 (bs, 1H, H3'), 6.96 (d, 1H, *J* = 4.8 Hz, H6), 8.42 (bs, 1H, H5), 9.60 (d, 1H, *J* = 170.0 Hz, H6'); ^{13}C NMR (125.77 MHz, DMSO-d_6) δ (ppm) 24.7, 25.4, 32.5, 48.5 (cyclohexyl), 106.0 (C6), 157.2 (C4), 160.2 (C5), 161.1 (C5'), 162.2 (C2); HRMS (ESI+): calcd for $C_{11}H_{18}N_5O^+$, $[M + H]^+$: 236.1506, found 236.1513.

2.7 | General procedure for the synthesis of pyrimidine derivatives 4a–q, 11a–f, and 12a–f

To a solution of hydrazide derivative (1 mmol, **3**: 0.214 g; **9**: 0.243 g; **10**: 0.253 g), in dimethyl sulfoxide (15 mL), was added the appropriate aldehyde or ketones (1 mmol) and hydrochloric acid (2 drops). The

TABLE 4 Selective index calculated for active compounds

	Selectivity index (SI)					
	L. amazonensis		*T. cruzi*			
Comp.	Pro-LLCMK$_2$[a]	Pro-L929[b]	Pro-J774A1[c]	Epi-LLCMK$_2$[d]	Epi-L929[e]	Epi-J774A1[f]
4b	–	–	>100	–	–	>8.3
4c	2.7	9.8	2.3	5.1	18.8	4.4
4f	>232	15.1	>232	–	–	–
4l	–	–	–	8.6	>29	5.9
5	4.1	3.1	3.4	3.2	2.4	2.6
6	5.5	4.4	4.3	5.1	4.2	4.1
7	6.4	4.8	5.4	4.7	3.5	4.0
8	5.0	3.9	3.2	3.4	2.6	2.2
11c	22.8	20.7	17.7	19.3	17.5	15.0
11f	22.7	22.6	19.8	19.3	19.2	16.8
12b	5.2	4.6	3.9	2.7	2.4	2.1
12f	17.3	16.3	13.1	6.4	6.1	4.9

Notes: –, not determined.
[a]Selectivity index: $CC_{50(\text{epithelial cells})}/IC_{50(\text{pro})}$.
[b]Selectivity index: $CC_{50(\text{fibroblast})}/IC_{50(\text{pro})}$.
[c]Selectivity index: $CC_{50(\text{macrophage})}/IC_{50(\text{pro})}$.
[d]Selectivity index: $CC_{50(\text{epithelial cells})}/IC_{50(\text{epi})}$.
[e]Selectivity index: $CC_{50(\text{fibroblast})}/IC_{50(\text{epi})}$.
[f]Selectivity index: $CC_{50(\text{macrophage})}/IC_{50(\text{epi})}$.

TABLE 5 Lipinski rule for most active compounds

Comp	MW (g/mol)	log P	H acceptors	H donors	Nrot	PSA
4b	390.49	5.13	5	1	5	67.25
4c	363.33	3.40	9	2	5	133.30
4f	317.35	2.27	6	1	4	80.14
4l	347.33	3.49	8	1	5	113.07
11c	392.38	3.29	10	3	7	145.32
11f	346.39	2.15	7	2	6	92.17
12b	425.58	5.90	6	2	7	79.27
12f	352.44	3.04	7	2	6	92.17

mixture was stirred at room temperature for 1 hr. The reaction product was precipitated with the addition of water (50 ml) and filtrated.

4-[(2*E*)-*N*′-(2-Thienylmethylene)hydrazinecarbonyl]-2-phenylpyrimidine (4a) is a white solid; 95% yield (0.293 g); mp 189.5–192.0; ^{1}H NMR (500.13 MHz, DMSO-d$_6$) δ (ppm) 7.19 (dd, 1H, *J* = 5.0, 3.6 Hz, H11'), 7.55 (dd, 1H, *J* = 3.7, 1.2 Hz, H13'), 7.58–7.60 (m, 3H, Ph), 7.75 (dt, 1H, *J* = 5.0, 1.0 Hz, H12'), 7.99 (d, 1H, *J* = 4.9 Hz, H6), 8.67–8.69 (m, 2H, Ph), 8.99 (s, 1H, H8'), 9.18 (d, 1H, *J* = 5.0 Hz, H5), 12.16 (s, 1H, H6'); ^{13}C NMR (125.77 MHz, DMSO-d$_6$) δ (ppm) 116.9 (C6), 128.0 (C11'), 128.5, 128.7 (Ph), 129.7 (C12'), 131.4 (Ph), 131.7 (C13'), 136.3 (Ph), 138.7 (C9'), 145.9 (C8'), 156.8 (C4), 159.3 (C5'), 160.2 (C5), 163.0 (C2); HRMS (ESI+): calcd for $C_{16}H_{13}N_4OS^+$, $[M + H]^+$: 309.0805, found 309.0821.

4-[(2E)-N′-(2-Thienylmethylene)hydrazinecarbonyl]-2-benzylamino-pyrimidine (11a) is an light yellow solid; 78% yield (0.263 g); mp 175.9–176.1; ^{1}H NMR (300.06 MHz, DMSO-d$_6$) δ (ppm) 4.70 (bs, 2H, H4'), 7.12 (d, 1H, *J* = 4.8 Hz, H6), 7.16 (dd, 1H, *J* = 5.0, 3.6 Hz, H11'), 7.19–7.24 (m, 1H, Ph), 7.29–7.39 (m, 4H, Ph), 7.49–7.50 (m, 1H, H13'), 7.71 (dt, 1H, *J* = 5.1, 1.0 Hz, H12'), 8.03 (bs, 1H, H3'), 8.53 (d, 1H, *J* = 4.8 Hz, H5), 8.80 (bs, 1H, H8'), 11.62 (bs, 1H, H6'); ^{13}C NMR (75.46 MHz, DMSO-d$_6$) δ (ppm) 44.1 (C4'), 107.2 (C6), 126.7, 127.4 (Ph), 128.1 (C11'), 128.3 (Ph), 129.6 (C12'), 131.6 (C13'), 138.8 (Ph), 140.2 (C9'), 145.1 (C8'), 156.6 (C4), 159.8 (C5'), 160.7 (C5), 161.8 (C2); HRMS (ESI+): calcd for $C_{17}H_{16}N_5OS^+$, $[M + H]^+$: 338.1070, found 338.1092.

4-[(2E)-N′-(2-Thienylmethylene)hydrazinecarbonyl]-2-cycloexylamino-pyrimidine (12a) is a white solid; 95% yield (0.313 g); mp 251.0–252.4; ^{1}H NMR (300.06 MHz, DMSO-d$_6$) δ (ppm) 1.13–1.32 (m, 5H, cyclohexyl), 1.58–1.90 (m, 5H, cyclohexyl), 3.91 (d, 1H, *J* = 60 Hz, cyclohexyl), 7.04 (d, 1H, *J* = 4.8 Hz, H6), 7.15 (dd, 1H,

J = 5.0 Hz, 3.6 Hz, H11'), 7.49–7.50 (m, 1H, H13'), 7.71 (dt, 1H, *J* = 5.0 Hz, H12'), 8.50 (d, 1H, *J* = 4.6 Hz, H5), 8.73 (bs, 1H, H8'), 11.62 (bs, 1H, H6'); ^{13}C NMR (75.46 MHz, DMSO-d_6) δ (ppm) 24.7, 25.4, 32.4, 49.3 (cyclohexyl), 106.6 (C6), 128.0 (C11'), 129.5 (C12'), 131.5 (C13'), 138.8 (C9'), 145.1 (C8'), 157.9 (C4), 160.1 (C5), 160.4 (C5'), 161.0 (C2); HRMS (ESI+): calcd for $C_{16}H_{20}N_5OS^+$, $[M + H]^+$: 330.1383, found 330.1405.

Characterization of all pyrimidine derivatives **4a–q**, **11a–f**, and **12a–f** can be found in the Supporting Information.

2.8 | Cell culture

Promastigotes forms of *Leishmania amazonensis* (WHOM/ BR/75/ JOSEFA strain) were cultured in Warren medium (brain heart infusion, hemin, and folic acid; pH 7.4) supplemented with 10% fetal bovine serum (FBS) at 25°C. Epimastigote forms of *Trypanosoma cruzi* (Y strain) were cultured in LIT medium (liver infusion tryptose; hemin, and folic acid; pH 7.4) supplemented with 10% FBS at 28°C. L929 fibroblast and $LLCMK_2$ (epithelial cells of kidney of *Macaca mulatta*) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, pH 7.2) supplemented with 10% FBS at 37°C in a 5% CO_2 atmosphere. J774A1 macrophages were cultured in RPMI-1640 (pH 7.2) medium supplemented with 10% FBS at 37°C in a 5% CO_2 atmosphere.

2.9 | Dilution of compounds

Stock solutions of the compounds were prepared in DMSO and then diluted in the respective medium. The groups (controls and treated) were tested with DMSO concentrations below 1%, with concentrations that do not affect viability of the protozoa and mammalian cells.

2.10 | Antiproliferative assay

Promastigote forms (1×10^6 parasites/ml) were cultured in 96-well plates in the presence and absence of different concentrations of compounds diluted in Warren medium supplemented with 10% FBS and incubated for 72 hr. Epimastigote forms (1×10^6 parasites/ml) were cultured in 96-well plates in the presence and absence of different concentrations of compounds diluted in LIT medium supplemented with 10% FBS and incubated for 96 hr. After treatment, the parasites were incubated with a solution of 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT; 0.5 mg/ml) and phenazine methanesulfonate activator (PMS; 0.06 mg/ml) in PBS for 4 hr. Then, the absorbance was read in a microplate reader (Bio Tek-Power Wave XS) at 450 nm. The percentage of viable parasites was calculated in relation to the control in order to determine the concentration that inhibits 50% of the protozoa (IC_{50}). The control groups of each experiment received the same experimental conditions of treated groups (cell concentration used, temperature, and incubation time).

2.11 | Cytotoxicity assay

Fibroblast L929 or $LLCMK_2$ cells (2.5×10^5 cells/ml) suspension was prepared in DMEM medium supplemented with 10% FBS and added to 96-well plates. Then, the plates were incubated at 37°C in a CO_2 atmosphere for 24 hr to obtain confluent cell growth. After incubation, cells were treated or not with different concentrations of compounds diluted in DMEM for 72 hr. Macrophage (5×10^5 cells/ml) suspension was prepared in RPMI-1640 medium supplemented with 10% FBS and added to 96-well plates. Then, the plates were incubated at 37°C in a CO_2 atmosphere for 24 hr to obtain confluent cell growth. After incubation, cells were treated or not with different concentrations of compounds diluted in RPMI-1640 for 48 hr. After treatment, medium was removed and cells were incubated with MTT (2 mg/ml) for 4 hr. Then, DMSO was added for solubilization of the formazan and analyzed with a reading microplate reader (BIO-TEK PowerWave XS spectrophotometer) at 392 nm. The percentage of viable cells was calculated in relation to the untreated control group to determine the cytotoxic concentration in 50% of the cells (CC_{50}). The control groups of each experiment received the same experimental conditions of treated groups (cell concentration used, temperature, CO_2, and incubation time).

2.12 | Rule of Five Lipinski

The compounds were also studied by applying the Rule of Five Lipinski (Lipinski, 2004; Molinspiration Cheminformatics Software [Online], 1986), making use of online free web cheminformatics software Molinspiration, the structures were drawn there and the values of polar surface area (PSA), molecular weight (MW), LogP, NroT, number of acceptor groups and hydrogen bond donors were obtained.

3 | RESULTS AND DISCUSSION

3.1 | Chemistry

To obtain the new target compounds, a series of reactions was performed (Scheme 1). For the construction of pyrimidine core **2** (Route A, Scheme 1), a cyclocondensation reaction between β-enamino ketone **1** (Hanzlowsky et al., 2003) and benzamidine hydrochloride, using sodium carbonate, MeCN reflux, for 24 hr, was employed (75%) (Rosa et al., 2008). In the next step, hydrazide derivative **3** (95%) was synthesized via an acyl nucleophilic substitution reaction between intermediate ester **2** and hydrazine monohydrate. From hydrazide-pyrimidine **3**, *N*-acylhydrazone derivatives **4a–q** (82–94%) were synthesized from the condensation reactions with different aldehydes and ketones (da Silva et al., 2016). To obtain different substituents on the C2 of the pyrimidine ring, the reaction between substrate **1** and *S*-methylisothiourea was tested using an aqueous solution of sodium carbonate (Route B, Scheme 1) (Aquino et al., 2017). However, the

desired product 4-(ethoxycarbonyl)-2-(methylthio)pyrimidine **5** was not obtained. For this reason, different solvents and bases were tested to optimize the reaction conditions, as indicated in Table 1. The best condition found for the cyclocondensation of β-enamino ketone **1** and *S*-methylisothiourea was obtained when the reaction was carried out in ethanol reflux, using triethylamine, for 24 hr (entry 7, Table 1). Next, the intermediate 4-(ethoxycarbonyl)-2-(methylsulfonyl)pyrimidine **6** was synthesized from the oxidation of the SMe group in compound **5** (Aquino et al., 2017); however, THF was employed as the reaction solvent. Thus, it was possible to obtain the desired product **6** with a 79% yield without purification steps. Posteriorly, the 4-(ethoxycarbonyl)-2-(amino)pyrimidine derivatives **7** (71%) and **8** (72%) were obtained from the reaction of **6** with benzylamine and cyclohexylamine, respectively (Aquino et al., 2017). Hydrazide derivatives **9** (90%), **10** (95%) and desired pyrimidines-*N*-acylhydrazone hybrids **11a–f** and **12a–f** (69–95%) were obtained by employing the same conditions used for the synthesis of **3** and **4**.

All synthesized compounds were characterized by ^{1}H and ^{13}C NMR spectroscopy, with the aid of the HSQC and HMBC contour maps. It was possible to observe in the ^{1}H NMR spectra that the majority of *N*-acylhydrazone derivatives were obtained in only one stereoisomeric form, probably the most stable form *E*, while compounds **4f**, **11e**, **11f**, **12e**, and **12f** were obtained as a mixture of *E*/*Z* forms, visible by duplication the signals, with a predominance of the most stable form *E* and ratio in DMSO-d_6 of 84/16, 73/27, 87/13, 86/14, and 97/03, respectively. Only one of the *N*-acylhydrazone derivatives was obtained in the *Z* form, the compound **4e**. The obtaining of compounds in the *Z* form occurs when it is possible to form an intramolecular hydrogen bond in the *N*-acylhydrazone subunit, as with the nitrogen atom of pyridine and the amidic hydrogen atom (Lopes et al., 2013).

3.2 | Evaluation of in vitro antiprotozoal activity

Synthesized pyrimidines were tested for in vitro antiprotozoal activities against *L. amazonensis* and *T. cruzi* and had their toxicity evaluated against three different cell lines: epithelial cell $LLCMK_2$, fibroblasts L929, and macrophage cells J774A1, as indicated in Tables 2 and 3. A selectivity index (SI) was calculated for each of the active compounds, as shown in Table 4.

The results obtained indicate that 2-phenyl pyrimidines derivatives containing ester and hydrazide moieties **2** and **3** showed no activity against *L. amazonensis*. On the other hand, some of the 4-*N*-acylhydrazone 2-phenylpyrimidine hybrids **4a–q** exhibited good values of inhibition. Among the compounds with R^1 containing neutral, electron-donating, and electron-withdrawing groups in the *para*-position and *ortho*-position, only compound **4c** showed an effect on antileishmanial activity, with the value of IC_{50} = 32.9 μM. Compounds **4p** and **4q** containing R^1 alkyl were not active against *L. amazonensis*. Among the compounds with the R^1 heteroaryl, the 2,2′-bithienyl group (**4a**) presented a value of IC_{50} = 12.0 μM, while the other heteroaryl derivatives were not active. However, the compound **4f** derivative from 2-acetyl pyridine had the best result of all series **4a–q**, with IC_{50} = 4.3 μM. Furthermore, **4f** exhibited a good selectivity index ($CC_{50(fibroblast)}/IC_{50(pro)}$) of 15.1 (Table 3).

To evaluate the influence of phenyl, benzyl and cyclohexyl groups attached on the 2-position of pyrimidine ring on activity, we performed an antiprotozoal assay of the compounds **5–12a–f**. Differently from ester derivative **2**, the compounds **5–8** were active, exhibiting an IC_{50} value range of 14.3–22.1 μM for antileishmanial activity and a SI value range of 3.1–6.4. However, hydrazide derivatives **9** and **10** were not active.

Among the compounds **11a–f**, the most active were **11c** and **11f**, with SI higher than 17. Regarding the compounds **12a–f**, the most active were **12b** and **12f**. However, **12b** was much more selective than **12f**.

In general, among these three series **4a–q**, **11a–f**, and **12a–f**, the best results were obtained for compounds containing a phenyl group on 2-position. Furthermore, the substituents 2-pyridinyl (R^1) and Me (R^2) on *N*-acylhydrazone were more effective toward antileishmanial activity. This activity could be directly related to the presence of a methyl group (R^2), since all derivatives with methyl were more active than the analogue without methyl (Feng et al., 2020). Another reason could be the presence of *E*/*Z* isomers, which has different chemical properties. It is also known that those isomers can interconvert to each other, and in the presence of polar solvents, like water, the isomer *E* is favored because of intermolecular hydrogen interaction with water; however, in this process of interconversion, a diazo-enamino tautomer is formed and could interact with its target (Landge et al., 2001).

Among the compounds of series **4a–q** evaluated for *T. cruzi*, the most active were **4c** (IC_{50} = 17.2 μM) and **4l** (IC_{50} = 33.6 μM). Both compounds presented a nitro group attached to the phenyl ring of *N*-acylhydrazone moiety. All ester derivatives **5–8** were active, exhibiting good IC_{50} value range 16.9–29.0 μM toward *T. cruzi*, while hydrazide derivatives **9** and **10** showed IC_{50} values greater than 100 μM. For series **11a–f**, the most active compounds for *T. cruzi* were **11c** (IC_{50} = 22.3 μM) and **11f** (IC_{50} = 29.7 μM), demonstrating similar behavior obtained for *L. amazonensis*. These compounds also exhibited good index selectivity (>15) for three cell lines evaluated (Table 4). Among compounds **12a–f**, the most active compound presented an IC_{50} value of 47.1 μM (**12b**), indicating that these series 2-cyclohexyl pyrimidine derivatives are not remarkably interesting for trypanocide activity.

The most active pyrimidine-*N*-acylhydrazone hybrids against *L. amazonensis* and *T. cruzi*. **4b**, **4c**, **4f**, **4l**, **11c**, **11f**, and **12b**, **12f**, were also analyzed for Lipinski's rule, or rule of five (Table 5), which evaluates the theoretical oral bioavailability, by specific parameters: molecular weight ≤500, log P ≤5, hydrogen acceptors ≤10, and hydrogen donors ≤5. The expansion of this rule also involves a number of rotation bonds (Nrot) ≤10 and a polar surface area (PSA) ≤140 $Å^2$, as indicated in Table 5. According to theoretical calculations, the compounds **4c**, **4f**, **4l**, **11c**, **11f**, and **12f** showed no violations regarding the Lipinski rules. On the other hand, compounds **4b** and **12b** containing a

2,2′-bithienyl in *N*-acylhydrazone moiety showed one violation of the Lipinski rule, with log P higher than 5, which means they may present poor oral bioavailability. All active compounds showed desirable values of Nrot and PSA, making compounds **4c**, **4f**, **4l**, **11f**, and **12f** useful for another drug development process.

4 | CONCLUSION

In summary, novel pyrimidine *N*-acylhydrazone hybrids were obtained through simple methods that allowed structural variations on the 2-position of the pyrimidine core and *N*-acylhydrazone subunit. in vitro evaluation of the antiprotozoal profile against *L. amazonensis* and *T. cruzi* revelated that eight hybrid compounds exhibited activity toward parasite proliferation and low cytotoxicity against host cell lines. The most active compound exhibited (IC_{50} = 4.3 μM) much higher selectivity toward *L. amazonensis* parasites when compared with the *T. cruzi* parasite and mammalian cells in culture. The substituent effects attached to the 2-position of the pyrimidine core on activity were observed, being that 2-phenyl pyrimidine derivatives were more active. The *N*-acylhydrazone subunit also played a significant role in the antiprotozoal activity. These findings corroborate the importance of the aza-heterocycle *N*-acylhydrazone hybrids to the development of potential antiparasitic agents. Furthermore, the pyrimidine *N*-acylhydrazone hybrid **4f** can be useful in future studies for optimization of drug candidate for neglected disease treatment.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

ORCID

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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